Bull. Mukogawa Women's Univ. Nat. Sci., **43**, 37－43(1995)
武庫川女子大紀要（自然科学）

Polymyxin P, Antibiotics from *Bacillus polymyxa* T-39

Fermentation, Isolation, Structure Elucidation and Antibacterial Activity

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Polymyxin P was isolated from a cultural broth of *Bacillus polymyxa* T-39 in 1969. Recently, we developed a new method to determine the chemical structure of *N*-fatty acyl peptide in combination with polymyxin acylase and a protein sequencer. Using this method, the chemical structures of polymyxin P_1 and P_2 were determined and their antibacterial activities were clarified.

Introduction

The discovery of polymyxins was announced in 1947 simultaneously by three different groups[1)-3)]. These antibiotics were isolated from cultures of *Bacillus polymyxa* and *B. aerosporus* and shown to have a good spectrum of activity towards Gram-negative bacteria. Further studies[4)] revealed that they might be a group of chemically and biologically related polypeptides. In an attempt to clarify the situation, collaborative work[5)] was done by the three groups on the chemical and biological properties. As a result, 'polymyxin' was accepted as the generic name for this group of antibiotics and alphabetical suffixes were used to name different types that differed in their amino acid composition. Antibiotics isolated from different strains of *B. polymyxa* are named polymyxin A, B, C[6)], D, E, K[7)], M[8)], P, S_1 and T_1[9)]. Similar antibiotics isolated from *Bacillus* species other than *Bacillus polymyxa* are named colistin[10)] (*Aerobacillus colistinus*), circulin[11)] (*B. circulans*), polymyxin F[12)] (*B. circulans* ATCC 31228) and polypeptin[13)] (*B. circulans*). These antibiotics consist of two components, with each containing 6-methyl octanoic acid or isooctanoic acid as the *N*-terminal acyl group. In 1969, Kimura *et al* isolated polymyxin P in our laboratory and reported this in a rapid short paper[14)]. The study of the chemical structure has continued and many polymyxin-type antibiotics have been found, as summarized in Table 1. Among them colistin and polymyxin B have been used clinically against bacterial infection. Recently, we developed a new method to determine the chemical structure of *N*-acyl peptide in combination with polymyxin acylase and protein sequencer. Using this method, we tried to reveal the chemical structure of polymyxin P_1 and P_2.

This paper describes the fermentation, isolation, structural elucidation and antibacterial activities of polymyxin P_1 and P_2.

Table 1. Comparison of constituent amino acids and fatty acids of polymyxins in literature

Antibiotic	Amino acid (ratio)						Fatty acid
	A_2bu	Thr	Leu	Phe	Ser	Ile	
Polymyxin A_1	5(L),1(D)	3(L)	1(D)				MOA
A_2	5(L),1(D)	3(L)	1(D)				IOA
Polymyxin M_1	6(L)	3(L)	1(D)				MOA
M_2	6(L)	3(L)	1(D)				IOA
Polymyxin K	6(L)	3(L)	1(D)				+[a]
Polymyxin B_1	6(L)	2(L)	1(L)	1(D)			MOA
B_2	6(L)	2(L)	1(L)	1(D)			IOA
Polymyxin C	+	+		+			+
Polymyxin P_1	6	3		1			MOA
P_2	6	3		1			IOA
Polymyxin D_1	5(L)	3(L)	1(L)		1(D)		MOA
D_2	5(L)	3(L)	1(L)		1(D)		IOA
Polymyxin E_1 (Colistin A)	6(L)	2(L)	1(L),1(D)				MOA
E_2 (Colistin B)	6(L)	2(L)	1(L),1(D)				IOA
Circurin A	6(L)	2(L)	1(D)			1(L)	MOA
B	6(L)	2(L)	1(D)			1(L)	IOA
Polymyxin S_1	5(L)	3(L)		1(D)	1(D)		MOA
Polymyxin T_1	6(L)	1(L)	2(L)	1(D)			MOA
Polymyxin F	5	1	2		1	1	MOA IOA

MOA : 6-methyl octanoic acid
IOA : isooctanoic acid
a : hydroxy fatty acid
D : D-configuration
L : L-configuration
+: Reported to be present

Materials and Methods

Fermentation

Bacillus polymyxa T-39 was isolated from a soil sample collected at Suma-ku, Kobe City, Japan.

A loopful of the organism on slant culture was transferred into four 500-ml Sakaguchi flasks each containing 100 ml of the seed medium. The medium contained 5% corn meal, 1% starch, 0.1% yeast extract, 0.5% ammonium sulfate, 0.1% potassium dihydrogenphosphate and 1% calcium carbonate. The pH was adjusted to 7.2 before sterilization. The flasks were incubated on a rotary shaker (150 rpm) for 1 day at 28℃. The resulting culture was transferred into four 2-liter sterile Sakaguchi flasks each containing 500 ml of the seed medium. It was cultured for 3 days at 28℃ with aeration and at an agitation speed of 120 rpm.

Isolation and purification

The isolation procedure outlined in *Fig.* 1 gave 2.1 g of the antibiotic acetate (light yellow powder) from 2 liters of fermentation broth. The antibiotic, named polymyxin P, was separated two fractions, polymyxin P_1 and P_2, by using countercurrent distribution (solvent system, n-BuOH / sec-BuOH / 0.1 N HCl = 6 / 30 / 40, transfer = 900 times). These two fractions were collected and distilled off, and the residue was lyophilized, yielding 1,080 mg of polymyxin P_1 hydrochloride and 270 mg of P_2 hydrochloride as a light yellow powder.

Polymyxin P_1 was purified by reversed-phase HPLC using a YMC-Pack SH-343-10-ODS column (25 x 2 cm i.d., Yamamura Chemical Institute, Kyoto, Japan), with isocratic elution with 0.1 M NaCl-HCl buff. (pH 2) in MeOH - H_2O (55 / 45) and a flow rate of 4.0 ml / minute,

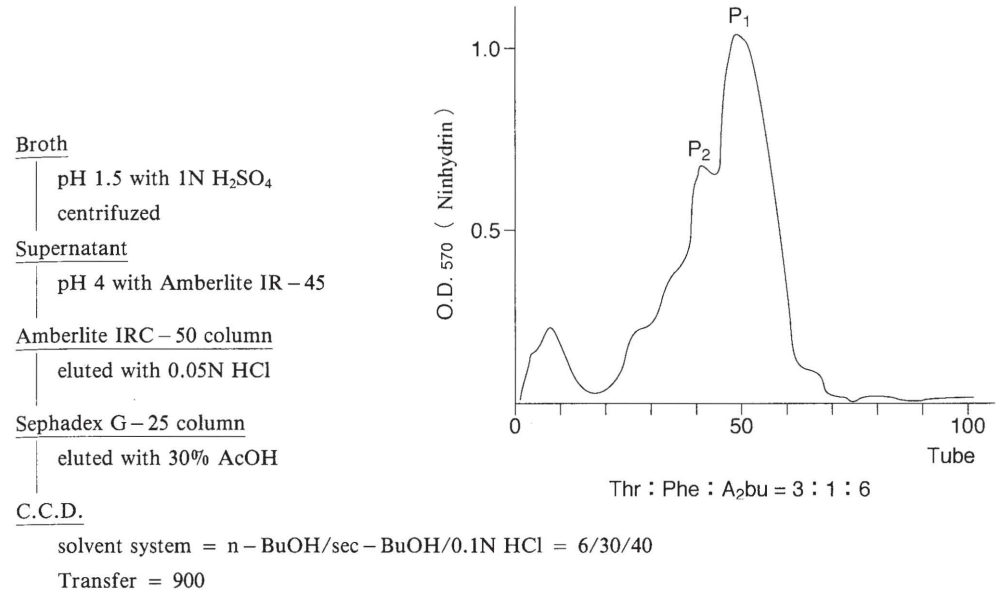


Fig. 1. Isolation of polymyxin P from *Bacillus polymyxa* T-39

and a variable-wavelength UV detector (875-UV, Japan Spectroscopic, Tokyo, Japan, set at 220 nm). The pH of the polymyxin P_1 fraction was raised to 4 with 0.1 M NaOH. The sodium chloride that formed was removed by applying Sephadex G-15 column (eluent : water). The desalted solution was lyophilized, yielding 570 mg of the main fraction of polymyxin P_1 hydrochloride as a white powder. Also, purification of polymyxin P_2 hydrochloride was carried out in the same manner as polymyxin P_1. One hundred fifty mg of polymyxin P_2 hydrochloride as a white powder was obtained.

Amino acid analysis

Polymyxin P_1 or P_2 hydrocloride, 100 μg, was hydrolyzed with 50 μl of constant-boiling hydrochloric acid at 110℃ for 18 hours. The hydrolyzate of polymyxin P_1 or P_2 was then analyzed with a Hitachi L-8500 automatic amino acid analyzer.

Preparation of deacyl polymyxin P_1 ($DAPP_1$) and P_2 ($DAPP_2$)

As described in previous papers[15, 16], polymyxin acylase (0.13 U : Wako Pure Chemicals, Ltd.) was added to 1% polymyxin P_1 or P_2 hydrochloride (0.1 ml) solution (pH 8.0) at 37℃ for 2 hours. After extraction of the liberated fatty acids, the water layer was adjusted to pH 4.0. The desalted sample was subjected to preparative column chromatography [column: YMC-Pack AM-324-10-ODS (30 x 1 cm i.d.), mobile phase : 0.01 M HCl in MeOH - H_2O (5 / 95)]. The main fractions were collected and after neutralizing the solution with 0.01 M NaOH, were lyophilized, yielding $DAPP_1$ or $DAPP_2$.

Amino acid sequence analysis

The peptide sequences of $DAPP_1$ and $DAPP_2$ were determined with a pulse liquid-phase protein sequencer (Applied Biosystems model 473A, Faster City, CA, U. S. A.) from approximately 100 pmol of material.

Gas chromatography of the fatty acids liberated from polymyxin P_1 and P_2

The ether fraction (see above) from polymyxin P_1 or P_2 was used to analyze fatty acids. Ether was removed by distillation, and the residue was

esterified with MeOH – BF_3 and analyzed in GC using the following conditions: an OV-1 column (2.1 m x 3.2 mm i.d.), the flow rate of 50 ml / minute for the carrier gas, an FID-detector.

FAB mass spectra

FAB mass spectra were obtained from a glycerol matrix with a JMS-DX 303 mass spectrometer (Jeol, Tokyo, Japan).

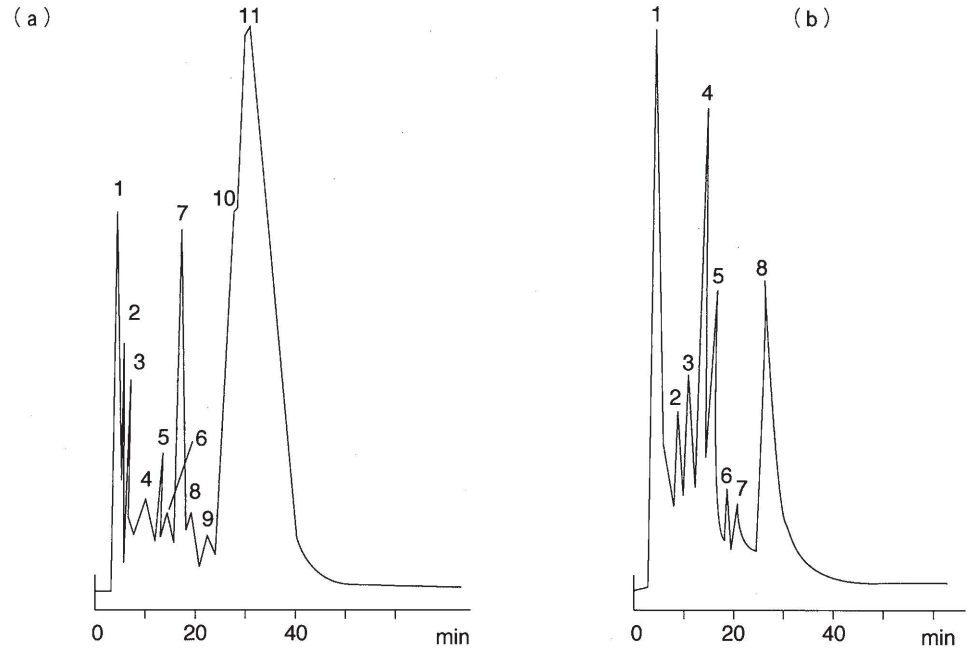
Antibacterial activity

The antibacterial potency determination was based on the agar-well method, modified from the official assay method for antibiotic preparation suggested by British Pharmacopoeia (1973). The minimum inhibitory concentrations of polymyxins P_1 and P_2 were determined by the twofold serial agar dilution method recommended by the Japan Society of Chemotherapy. The medium was Muller-Hinton agar.

Results and Discussion

In the early stage, a homogeneous polymyxin purified by normal phase column chromatography could be separated into two or three components by a countercurrent distribution technique. Studies of the chemical structure of the polymyxin antibiotics were carried out together with the preparation of penta *N*-2,4-dinitrophenyl polymyxins and their partial hydrolyzate. We had previously separated polymyxin P into polymyxin P_1 and P_2 by countercurrent distribution as shown in *Fig.* 1, and estimated their cyclic peptide structure by classical methods. These methods required large amounts of sample and long operation times. After that, it was found that polymyxin P_1 and polymyxin P_2 could be easily separated by reversed phase (ODS) chromatography. Furthermore, analytical ODS column chromatography could separate the polymyxin P_1 sample (which had been prepared by countercurrent distribution) into 11 peaks (*Fig.* 2 (a)) and also the polymyxin P_2 sample into 8 peaks (*Fig.* 2(b)). Table 2 shows the amino acid ratio constituting each peak. The ratio of A_2bu (2,4-diamino butyric acid) : Thr : Phe for peak 5 – 11 in polymyxin P_1 was approximately 6 : 3 : 1, respectively, and was the same for peak 2 – 8 in polymyxin P_2. Peak 11 was the main peak of polymyxin P_1 (*Fig.* 2(a)) and peak 4 for polymyxin P_2. (Fig. 2(b)) As shown in Table 1, the amino acids constituting polymyxin C[6] were the same as those of polymyxin P, but the amino acid ratio of polymyxin C has not been reported. As given below, the amino acid sequences of these fractionated compounds were the same. These results suggest that some adduct may be included by non-covalent bond in the cyclic peptide structure of each compound. The molecular ion $[M+H]^+$ of polymyxin P_1 by FAB-mass was 1191 and that of polymyxin P_2 was 1177. (*Fig.* 3) These values agreed with the expected chemical structure of polymyxin P_1 and P_2, respectively. Some difference was observed in the mode of fragmentation between polymyxin P_1 and P_2. We suppose that some adduct in polymyxin P_1 is different from that of polymyxin P_2.

Several years ago, we found a new enzyme, polymyxin acylase which hydrolyzes not only the *N*-terminal fatty acyl group of polymyxins, but also that of many *N*-fatty acyl peptides. Using this polymyxin acylase, we prepared $DAPP_1$ and $DAPP_2$. The amino acid sequences of these deacylated peptides were both $A_2bu \rightarrow Thr \rightarrow A_2bu \rightarrow$ none $\rightarrow A_2bu \rightarrow Phe \rightarrow Thr \rightarrow A_2bu \rightarrow A_2bu \rightarrow Thr$. This structure corresponded to that expected for $DAPP_1$ and $DAPP_2$ by the classical method. The peptide sequence of other fractions having the same amino acid ratio (A_2bu : Thr : Phe = 6 : 3 : 1) was the same as that of main fraction. In this sequence, no amino acid was detected in the fourth cycle, indicating that polymyxin P had preserved its cyclic structure (the amide linkage between the γ-amino group of the A_2bu residue of the fourth cycle and the carboxyl group of *C*-terminal Thr). Gas chromatographic analysis of the liberated fatty acid from polymyxin P_1 and P_2 gave different results. The fatty acid from polymyxin P_1 was optically active and was identical with authentic 6-methyl octanoic acid. On



Column: YMC-Pack AM-312 (6×150 mm)
Mobile phase: 0.1 M NaCl-HCl buff(pH 2.2) : MeOH = 55/45

Fig. 2. Purification of polymyxin P_1 (a) and P_2 (b)

Table 2. Amino acid analysis of polymyxin P_1 and P_2

Polymyxin P_1 Peak No.	A_2bu	Thr	Phe	Polymyxin P_2 Peak No.	A_2bu	Thr	Phe
1	3.61	1.40	1.00	1	3.75	1.92	1.00
2	3.08	1.61	1.00	2	7.07	2.58	1.00
3	3.19	1.57	1.00	3	7.28	2.89	1.00
4	3.38	1.57	1.00	**4**	**6.83**	**3.01**	**1.00**
5	6.57	2.91	1.00	5	7.18	2.99	1.00
6	6.30	2.78	1.00	6	6.18	2.12	1.00
7	6.30	2.79	1.00	7	5.93	2.45	1.00
8	6.46	2.93	1.00	8	6.60	3.05	1.00
9	6.44	2.88	1.00				
10	5.90	2.73	1.00				
11	**6.52**	**2.86**	**1.00**				

the other hand, that from polymyxin P_2 was optically inactive and identical with authentic isooctanoic acid. Thus, the chemical structures of polymyxin P_1 and P_2 were determined as shown in *Fig.* 4.

The antibacterial spectra of polymyxin P_1 and P_2 were similar to those of polymyxin B. (Table 3) They were strongly active against Gram-negative bacteria, such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, and the

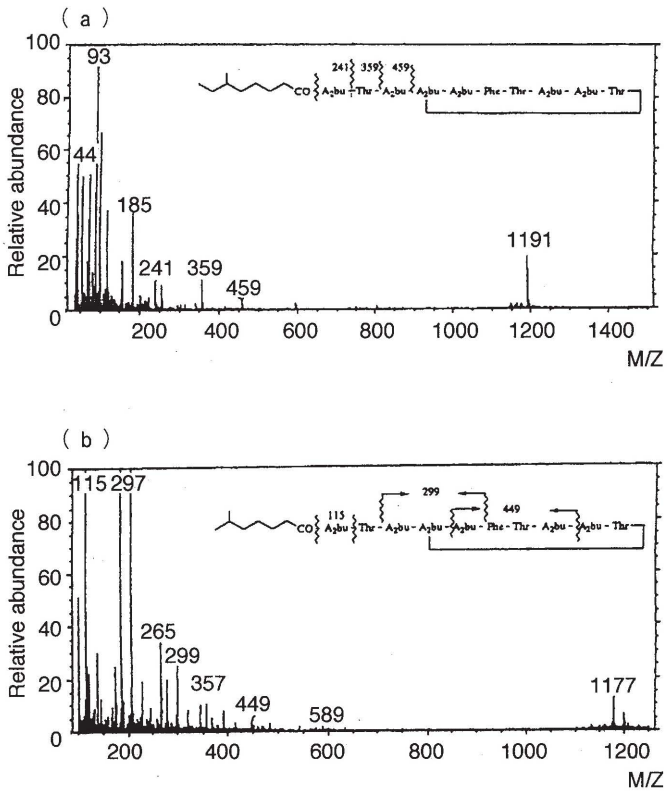


Fig. 3. FAB–mass spectra of polymyxin P_1 (a) and P_2 (b)

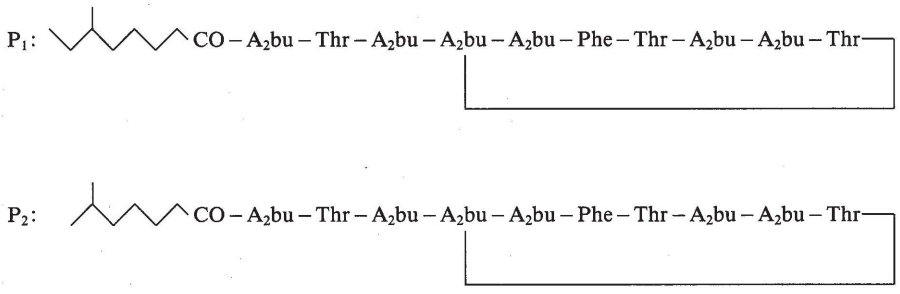


Fig. 4. Structure of polymyxin P_1 and P_2

antibacterial potency of polymyxin P_1 was higher than that of polymyxin P_2.

In this work, we developed a new method to determine the chemical structure of polymyxin antibiotics using a small amount of sample (1 mg) with a short operation time (about one day). This method should be very useful for determining the chemical structure of many *N*-acyl cyclic peptides.

Table 3. Antimicrobial spectrum and potencies of polymyxin P_1 and P_2

Test organism	MIC (μg/ml)			
	P_1	P_2	Complex	Polymyxin B
Escherichia coli	1.56	3.12	0.78	1.56
Klebsiella pneumoniae	1.56	3.12	0.78	0.39
Shigella sonnei I	0.78	0.78	0.78	0.39
Pseudomonas aeruginosa	12.5	6.25	3.12	3.12
P. aeruginosa P_2	12.5	6.25	3.12	3.12
P. aeruginosa P_3	12.5	6.25	3.12	3.12
Staphyrococcus aureus	100	>100	100	100
Bacillus subtilis	100	100	100	100
Potency (U/mg)	8,090	1,800	6,760	8,000

Medium : Modified Müller Hinton agar

Inoculum : One loopful of 10^6 cfu/ml suspension of each strain.

Potency (U/mg) : expressed as polymyxin B units.

Acknowledgments

We are grateful to Ms. R. Nousou and Ms. N. Hayashi for their technical assistance. We also thank Professor Judy Noguchi for her advice in preparing the manuscript.

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